

MEF up-regulates human  $\beta$ -defensin 2 expression in epithelial cellsZhuo Lu<sup>a</sup>, Kyoung-Ah Kim<sup>a</sup>, Mary Ann Suico<sup>a</sup>, Tsuyoshi Shuto<sup>a</sup>, Jian-Dong Li<sup>b</sup>, Hirofumi Kai<sup>a,\*</sup><sup>a</sup>Department of Molecular Medicine, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan<sup>b</sup>Gonda Department of Cell and Molecular Biology, House Ear Institute, University of Southern California, Los Angeles, CA 90057, USA

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**Abstract** Human  $\beta$ -defensin 2 (HBD2), an antimicrobial peptide, is widely expressed in epithelial tissues and displays a potent killing activity in response to the invasiveness of a wide range of microorganisms and the stimulation of various molecules. Myeloid ELF-1-like factor (MEF) has been reported to be involved in innate immunity responses, such as activation of perforin and lysozyme transcription. The role of MEF in the transcription regulation of HBD2, however, is unknown. Here, we show that MEF not only activated HBD2 promoter activity, but also increased the endogenous HBD2 transcription level. Moreover, the activated HBD2 promoter activity was attenuated by the antisense MEF RNA input and the loss of the ETS binding site (EBS: GGAA core sequence) in the HBD2 promoter. The interaction between the EBS and MEF protein was further confirmed by electrophoretic mobility shift assay. Thus, our data indicate that MEF may play an important role in regulating HBD2 expression in epithelial cells.

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**Key words:** Myeloid ELF-1-like factor; Human  $\beta$ -defensin 2; ETS binding site

## 1. Introduction

Antimicrobial molecules secreted by various tissues constitute an essential component of innate immunity [1,2]. Defensins are extensively characterized antimicrobial peptides and have a broad spectrum of antimicrobial activity against bacteria, yeasts and fungi. Defensins are divided into  $\alpha$ - and  $\beta$ -defensin subfamilies according to the positions of six highly conserved cysteine residues that participate in disulfide linkages [3]. Among the four human  $\beta$ -defensins (HBD1–4) characterized in various epithelial tissues, HBD2 is detected in epithelial cells of the skin, lung, gingiva, trachea and reproductive tract. Its expression is inducible in response to the invasiveness of yeast and both Gram-negative and Gram-positive bacteria [4,5], and stimulation by lipopolysaccharide [6–8] and a number of proinflammatory cytokines [9–12]. Multiple pathways, including the NF- $\kappa$ B pathway [4–6,13] and the mitogen-activated protein kinase pathway [14,15], have been reported to be involved in HBD2 regulation.

Genomic analysis of HBD2 revealed a promoter region

containing several putative transcription factor binding sites, including NF- $\kappa$ B, activator protein (AP)-1, AP-2, and NF-interleukin (IL)-6, which are known to be involved in the regulation of inflammatory responses [9]. Additionally, the putative binding site (core sequence GGAA) of ETS transcription factors (EBS) was also identified in this promoter, but it has not been investigated whether the ETS family of transcriptional factors is involved in HBD2 regulation.

The ETS family of transcription factors is characterized by an 85-amino-acid ETS domain that recognizes a core sequence GGAA or TTCC and thereby specifically binds to DNA [16]. The ETS family of transcription factors plays important roles in the development and function of multiple mammalian cell types. Its functions in epithelial cells have been a subject of active recent investigations. Myeloid ELF-1-like factor (MEF), an ETS transcriptional factor [17], is located on chromosome Xq26.1 with an observed protein size about 98 kDa. It has potent transcriptional activating effects on genes expressed in both lymphoid and myeloid cells. A study by Miyazaki et al. [18] demonstrated that the transcriptional activity of MEF varies during different phases of the cell cycle in hematopoietic cells. MEF not only possesses tumor-suppressive capability through inhibiting the transcription of some tumorigenic factors, such as matrix metalloproteinase (MMP)-9 and IL-8 [19], but also plays an essential role in innate immunity. It is required in epithelial cells to regulate the expression of lysozyme, which is an important component of innate immunity against common pathogens [20]. Furthermore, MEF also directly transactivates perforin in NK cells. MEF<sup>−/−</sup> mice have a profound reduction in the number of NK-T and NK cells. Purified MEF<sup>−/−</sup> NK cells secrete only minimal amounts of interferon- $\gamma$  and cannot be targeted to tumor. These facts indicate a specific role of MEF in the development and function of NK cells and in innate immunity [21].

In this study, we sought to investigate whether MEF affects the transcriptional activity and expression of HBD2 in human epithelial cells. Our results indicate that MEF is a potential activating factor for transcriptional regulation of HBD2.

## 2. Materials and methods

### 2.1. Cell culture

A549 cells (human pulmonary adenocarcinoma cells), stably MEF-transfected A549 cells, NIH3T3 cells (mouse fibroblast cells) and HEK293 cells (human kidney cells) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO<sub>2</sub> and 95% air atmosphere.

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Table 1  
Sequences of oligonucleotides used for PCR, RT-PCR

Name	Sequence (5'–3')
HBD2(–247)	GAGGAATTTTCTGGTCCCAAG
HBD2(–247)mut	AAGAGCAGGAGCTAGGGATTTTCT
HBD2(–89) <i>XhoI</i>	CCGCTCGAGAATACCAGTTCTGAACCTCA
HBD2(+22+44)	CCATGAGGGTCTTGATCTCCTC
Trans Oligo <i>Scal/StuI</i>	GTGACTGGTGAGGCCTCAACCAAGTC
HBD2-1A	GGAGCCCTTTCTGAATCCGCA
HBD2-1S	CCCAGCCATCAGCCATGAGGGT
GAPDH-up	GGAGCATGTGAATGCCATC
GAPDH-down	GGATGACAAGCAGAAAGTC

HeLa cells (human cervical adenocarcinoma cells) were grown in minimum essential medium supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO<sub>2</sub> and 95% air atmosphere.

## 2.2. Preparation of plasmid DNA

HBD2(–247) was cloned by PCR using a Genome Walker Kit (Clontech). A 5' primer, HBD2(–247), and a 3' primer, HBD2(+22+44), were used for polymerase chain reaction (PCR). The PCR product was cloned into pCR2.1 vector using the Original TA cloning kit (Invitrogen). After confirming the sequence, it was cloned into the *SacI* and *XhoI* sites of pGL2-basic vector, a promoter-less luciferase expression plasmid.

HBD2(–247)mut is a wild type HBD2(–247) construct with a mutant EBS. It was created from HBD2(–247) using a Transformer site-directed mutagenesis kit (Clontech). A mutation primer, HBD2(–247)mut, which contains a mutant EBS, and a selection primer, Trans Oligo *Scal/StuI*, were used.

HBD2(–89) was prepared by PCR using HBD2(–247) as a template. A 5' primer, HBD2(–89)*XhoI*, and a 3' primer, HBD2(+22+44), were used. The PCR product was cloned into the *XhoI* sites of pGL2-basic vector.

The sequences of primers used in this study are shown in Table 1. The expressing vectors of ETS family transcription factors and antisense MEF were prepared as previously described [20]. They were cloned into mammalian expression vector pCB6.

## 2.3. Transfection and luciferase assay

Transient transfections of plasmid DNA were performed with TransIT-LT1 (Panvera) according to the manufacturer's recommendations. Briefly, TransIT-LT1 and Opti-MEM were mixed thoroughly and incubated at room temperature for 15 min. Then DNA was added to the diluted TransIT-LT1 reagent (ratio: 1 µg DNA/3 µl LT1). After being mixed gently, the mixture was incubated at room temperature for 15 min. Then the mixture was directly added to the cells cultured on 24-well plates, without changing the medium. Forty-eight hours after transfection, the medium was removed and cells were harvested for luciferase assay. Cotransfection of the pRL-CMV (Promega), which expresses *Renilla* luciferase, verified that differences in firefly luciferase reporter gene expression were not due to differences in transfection efficiency. Luciferase activity was measured as previously described [20]. Values are shown as means ± S.E.M. ( $n = 4$ ). To normalize expression levels of the transcription factors, we measured mRNA by Northern blotting using the transcribed sequence of pCB6 as a probe.

## 2.4. RNA preparation and reverse transcription (RT)-PCR

Isogen (Nippon Gene) was used for extracting total RNA from cells according to the manufacturer's instructions. The concentration of total RNA obtained above was determined by measuring the UV absorbance (260 nm). RNA with high purity (OD<sub>260</sub>/OD<sub>280</sub> ≥ 1.80) was used for experiments.

A549 cells and stably or transiently MEF-transfected A549 cells were grown to about full confluence after 48 h incubation. Cells were harvested and total RNA was extracted. RT-PCR was performed with 0.5 µg RNA using a RNA PCR kit (Takara) according to the manufacturer's instructions: 30°C for 10 min, 42°C for 60 min, 99°C for 5 min, and 5°C for 5 min for reverse transcription; 95°C for 1 min, 60°C for 1 min, 72°C for 1.5 min, 40 cycles. Primers HBD2-1A and HBD2-1S were used. Expression of glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) was checked as an internal control. The 5' primer GAPDH-up and the 3' primer GAPDH-down were used.

## 2.5. Western blotting

Whole cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 7.5% gel, then the proteins were transferred to a polyvinylidene difluoride membrane. After blocking with 5% skim milk at 4°C overnight, membranes were incubated with affinity-purified rabbit antisera to MEF (1/200; TransGenic) at room temperature for 2 h. The membranes were subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1/10000; Seikagaku) at room temperature for 1 h. Finally chemiluminescence was detected using the ECL kit (Amersham Life Science).

## 2.6. Preparation of nuclear extract

A549 cells ( $1 \times 10^6$ ) were washed, collected and pelleted with phosphate-buffered saline by centrifugation at  $1500 \times g$  for 5 min, at 4°C. The pellet was resuspended in 400 µl of cold buffer containing 10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA (pH 8.0), 0.1 mM EGTA (pH 8.0), 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride by gentle pipetting. The cells were then allowed to swell on ice for 15 min, after which 25 µl of 10% Nonidet P-40 solution was added, and the tube was vigorously vortexed for 10 s. The homogenate was centrifuged at 15000 rpm for 1 min, at 4°C. The nuclear pellet was resuspended in 50 µl of ice-cold buffer containing 20 mM HEPES-KOH (pH 7.9), 0.4 M NaCl, 1 mM EDTA (pH 8.0), 1 mM EGTA (pH 8.0), 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride, the tube was vigorously vortexed for 15 min at 4°C. Then the nuclear extract was centrifuged at 15000 rpm for 5 min, at 4°C and the clear supernatant was collected, quickly frozen in liquid nitrogen, and stored at –80°C until further use. Protein concentration was determined using the Bradford assay (Bio-Rad) and bovine serum albumin as standard (Sigma).

## 2.7. Electrophoretic mobility shift assay (EMSA)

The double-stranded oligonucleotides EBS(wt), EBS(mut), and EBS-S(mut\*) were synthesized for use in the EMSA experiments. The sense sequences of each pair are listed in Fig. 4A. The probe EBS(wt) was labeled with [ $\gamma$ -<sup>32</sup>P]ATP (22 TBq/mmol) using T4 polynucleotide kinase (Takara). Pre-incubation of 5–10 µg nuclear extract with 2 µg poly(dI-dC) (Amersham Pharmacia Biotech), excess unlabeled EBS(wt), EBS(mut) or EBS-S(mut\*), antibody to MEF or ETS2 (Santa Cruz Biotechnology) or ESE2 (Santa Cruz Biotechnology) was carried out on ice for 30 min in buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.05% Nonidet P-40, 5% glycerol and 1 mg/ml bovine serum albumin. Radiolabeled oligonucleotide probes (0.04 pmol,  $5 \times 10^4$  cpm) were then added to the reaction mixtures and incubated at room temperature for 30 min. The reaction products were then analyzed by electrophoresis on a 5.5% polyacrylamide gel, followed by BAS2000.

## 3. Results

### 3.1. MEF up-regulates HBD2 promoter activity

HBD2(–247), the promoter construct we used, contains the main elements responsive to inflammatory factors and the EBS (Fig. 1A). To determine the role of MEF in HBD2 regulation, we first examined the effects of expressing ETS factors on HBD2(–247) promoter activity in the human lung epithelial cell line A549. MEF activated the HBD2(–247) promoter more strongly than the other ETS factors in A549 cells (Fig. 1B). A similar result was also observed in HeLa cells. In addition, in the mouse fibroblast cell line (NIH3T3) (Fig. 1C), which does not endogenously express MEF, MEF transfection induced much higher HBD2(–247) activity. The positive involvement of MEF was further confirmed by transfecting the antisense MEF RNA construct, which blocked the endogenous MEF. As shown in Fig. 1D,E, HBD2(–247) transactivity was inhibited by transfecting antisense MEF RNA into stably MEF-transfected A549 or HEK293 cells, which have a high background of MEF expression. The effi-

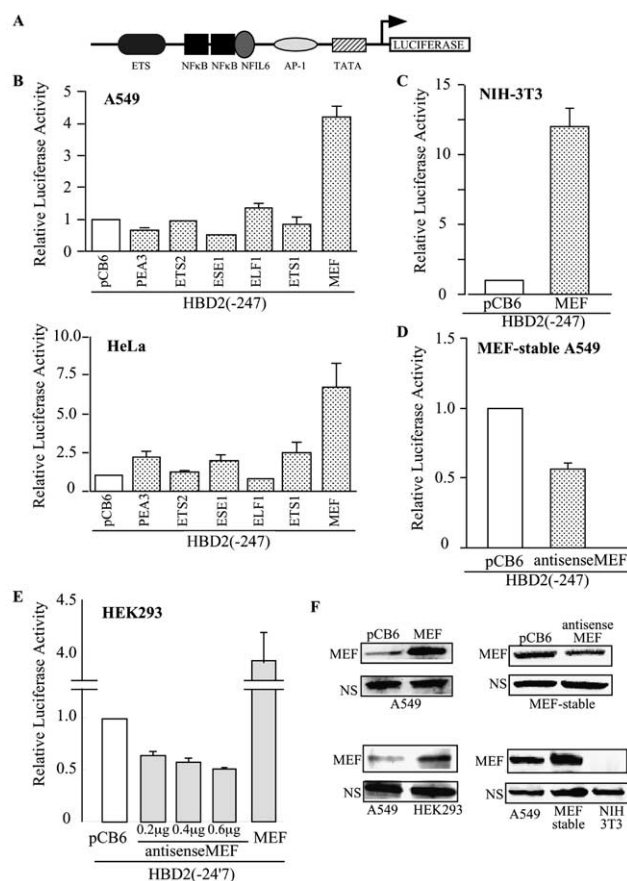


Fig. 1. MEF up-regulates HBD2 promoter activity. A: Schematic representation of HBD2(–247) containing the indicated putative *cis*-elements. B–E: A549 cells, stably MEF-transfected A549 cells, HeLa cells, NIH-3T3 cells and HEK293 cells were transiently transfected with the HBD2 promoter construct HBD2(–247) (0.2 μg/well) and the indicated ETS transcription factors (0.4 μg/well, if the DNA amount is not indicated in the figure) or antisense MEF (0.4 μg/well), using TransIT-LT1. Forty-eight hours after transfection, cells were harvested for luciferase assay. The fold transactivity of HBD2(–247) with empty pCB6 is assigned the value of 1 (open bars). F: MEF expression at protein level was examined by Western blotting in different cells. For transient transfection, A549 cells were transfected with DNA (indicated at the top of each panel) using TransIT-LT1 at the ratio 1 μg DNA/3 μl LT1 in 6-well plates. The stably MEF-transfected A549 cells are indicated as MEF-stable. A non-specific band (NS) serves as a loading control.

ciency of DNA transfection and MEF expression levels in each cell line were examined by Western blotting (Fig. 1F).

### 3.2. MEF up-regulates endogenous HBD2 expression

To test the effect of MEF on the endogenous transcription level of HBD2, we investigated the HBD2 mRNA level in transiently and stably MEF-transfected A549 cells (Fig. 2). Both of them showed a higher HBD2 mRNA level than un-

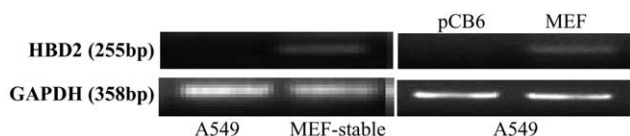


Fig. 2. HBD2 mRNA levels in original A549 cells and stably and transiently MEF-transfected A549 cells. RT-PCR was performed according to the manufacturer's instructions (RNA PCR kit, Takara).

transfected A549 cells, suggesting that MEF also mediates the transactivation of the endogenous HBD2 gene in A549 cells.

### 3.3. The transactivity of MEF on HBD2 promoter is EBS-dependent

The experimental results shown above indicate that MEF up-regulates HBD2 proximal promoter activity. To investigate whether the EBS in HBD2(–247) is involved in MEF regulation, we first developed a shorter promoter construct, HBD2(–89), in which EBS was deleted. Interestingly, MEF no longer potently transactivated its promoter activity (Fig. 3A). Next, we introduced nucleotide mutations into EBS in HBD2(–247) and then compared the transactivity of MEF on this mutant promoter and wild type promoter. As shown in Fig. 3B, MEF increased wild type HBD2(–247) activity in a dose-dependent manner, and this increase was greatly attenuated by the loss of EBS, indicating that MEF may exert its transactivity by binding to the EBS in HBD2(–247). It should be noted that MEF still retained some transactivating ability on HBD2(–247)mut, although to a lesser extent in comparison with wild type construct. Thus, it is likely that MEF may simultaneously regulate this promoter through other unidentified mechanism(s).

### 3.4. MEF binds to EBS in HBD2 promoter

Having demonstrated its ability to transactivate HBD2 pro-

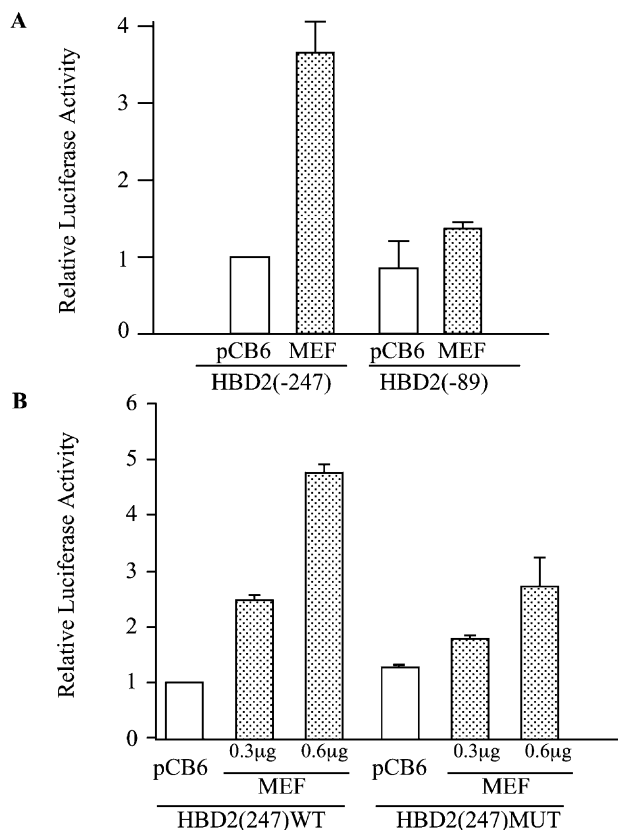


Fig. 3. MEF loses transcriptional activity on HBD2 promoter without EBS. A549 cells were transiently transfected with the HBD2 promoter construct HBD2(–247) or HBD2(–89) (0.2 μg/well) and MEF (0.4 μg/well, if the DNA amount is not indicated in the figure) using TransIT-LT1. Forty-eight hours after transfection, cells were harvested for luciferase assay.



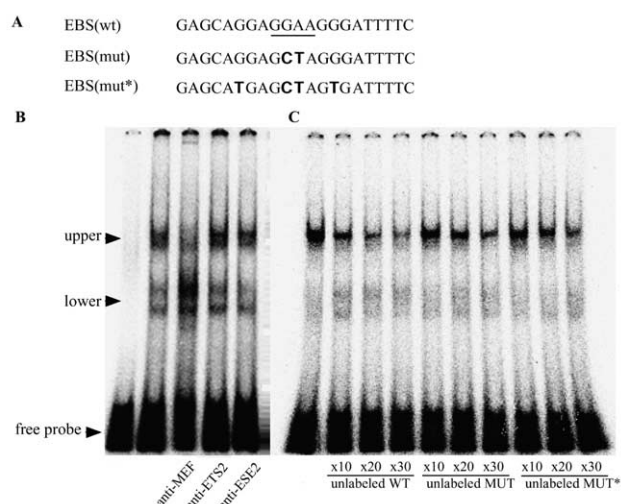


Fig. 4. Binding of MEF to EBS in HBD2 promoter. EBS(wt) probe (0.04 pmol) was used for each lane. A: Sequences of oligonucleotide probe and competitors. EBS(mut) and EBS(mut\*) have mutated nucleotides indicated in boldface letters. B: Anti-MEF (0.12  $\mu$ g), anti-ETS2 (0.2  $\mu$ g) and anti-ESE2 (0.2  $\mu$ g) antibodies were individually used for antibody reaction. C: As indicated, compared to the probe, an excess of unlabeled competitor oligonucleotides was added to the reaction mixture for the competitor experiment.

motor activity, we next examined whether MEF protein directly interacts with the proximal EBS by using the  $\gamma$ - $^{32}$ P-labeled HBD2 promoter sequence EBS(wt) as a probe (Fig. 4A), which contains intact EBS. EMSA (Fig. 4B) indicated that the upper band resulted from the binding between MEF protein and probe, because the antibody against MEF attenuated this band. The interaction of the polyclonal antibody with MEF protein may interfere with the binding of MEF with probe DNA, so that the supershifted band could not be observed. The lower bands are supposed to correspond to other ETS family factors competing for the EBS or some proteins having affinity to the probe sequence, because these bands became stronger after the MEF binding was blocked by its antibody. The antibodies to ETS2 and ESE2, used as controls, had no effect on any of these bands. In addition, we also performed the competitor assay using unlabeled probe sequence HBD(wt) and sequences with EBS mutations, EBS(mut) and EBS(mut\*) (Fig. 4C). In EBS(mut\*), besides the identified EBS, the other two sites similar to the EBS sequence, GGAG and GGAT, were also mutated (Fig. 4A). Compared to EBS(mut) and EBS(mut\*), unlabeled EBS(wt) attenuated the upper band more strongly, thereby confirming the binding between the probe with wild type EBS and MEF protein.

#### 4. Discussion

Defensins comprise a group of biological effectors, which can protect various tissues from microbial invasion and infection through direct killing as well as recruiting immune cells (T cells and dendritic cells). Thus defensins play a critical role in the innate immunity system and the link between innate and acquired immunities. Among the defensin family, the induction of HBD2 has been extensively studied recently. A variety of exogenous stimuli and physiological factors have been shown to induce HBD2. The NF- $\kappa$ B pathway appears

to be the best-known pathway involved in transducing these signals to the transcriptional regulation of HBD2 [6,22]. In addition, it is reported that AP-1 and intracellular calcium are also involved in HBD2 expression in airway epithelial cells [23].

The data presented here indicate MEF enhanced not only HBD2 promoter activity but also its endogenous transcription. In support of this notion, expressing antisense MEF RNA attenuated HBD2 promoter activity in both stably MEF-transfected cells and those without exogenous input of MEF. Furthermore, the EBS in HBD2 promoter appears to be essential for its binding with MEF protein and the consequent promoter transactivation. Together, these results indicate that MEF acts as a novel transactivator of HBD2 in epithelial cells, which functions through a mechanism independent of NF- $\kappa$ B. Thus MEF may have the potential to enhance basic HBD2 expression under non-irritated physiological conditions.

MEF belongs to the ETS transcription factor family, which plays an important role in cell growth, death and differentiation. It was revealed to suppress the promoter activities of MMP-9 and IL-8 genes, thus was thought to be a tumor suppressor gene that is down-regulated by methylation in cancer cells [19]. Although MEF constitutively localizes in the nucleus [24], its activity may vary under different circumstances or in different cell contexts. A recent study suggested that, in hematopoietic cells, its transcriptional activity was largely restricted to the G1 phase of the cell cycle [18]. This phenomenon is due to its change in phosphorylation status, which may also occur in epithelial cells. Our study also indicates that MEF may respond to some exogenous stresses through a change of its subnuclear localization, which, in turn, results in a change of its transcriptional activity (unpublished data).

As to the role of MEF in the immune system, deficiency of MEF expression in mice resulted in a significant reduction of the number of NK-T cells in thymus. Simultaneously NK cell development and function and perforin transcription were also impaired. Thus it is clear that MEF is a key transcriptional regulator in innate immune cells [21]. On the other hand, in epithelial cells, based on our previous study, MEF positively regulates the antimicrobial protein, lysozyme, at the transcriptional level [20]. Therefore, our present data provide evidence of the linkage between MEF and the antimicrobial peptide HBD2. Taken together, it is implied that MEF also plays important roles in innate immunity of epithelial cells.

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